Inhibition of Calcium/Calmodulin-Dependent Protein Kinase Kinase β and Calcium/Calmodulin-Dependent Protein Kinase IV Is Detrimental in Cerebral Ischemia

Louise D. McCullough, MD, PhD; Sami Tarabishy, BS; Lin Liu, PhD; Sharon Benashski, MS; Yan Xu, MD; Thomas Ribar, BS; Anthony Means, PhD; Jun Li, PhD

Background and Purpose—Elevation of intracellular calcium was traditionally thought to be detrimental in stroke pathology. However, clinical trials testing treatments that block calcium signaling have failed to improve outcomes in ischemic stroke. Emerging data suggest that calcium may also trigger endogenous protective pathways after stroke. Calcium/calmodulin-dependent protein kinase kinase (CaMKK) is a major kinase activated by rising intracellular calcium. Compelling evidence has suggested that CaMKK and its downstream kinase CaMK IV are critical in neuronal survival when cells are under ischemic stress. We examined the functional role of CaMKK/CaMK IV signaling in stroke.

Methods—We used a middle cerebral artery occlusion model in mice.

Results—Our data demonstrated that pharmacological and genetic inhibition of CaMKK aggravated stroke injury. Additionally, deletion of CaMKK β, one of the 2 CaMKK isoforms, reduced CaMK IV activation, and CaMK IV deletion in mice worsened stroke outcome. Finally, CaMKK β or CaMK IV knockout mouse had exacerbated blood–brain barrier disruption evidenced by increased hemorrhagic transformation and activation of matrix metalloproteinase. We observed transcriptional inactivation including reduced levels of histone deacetylase 4 phosphorylation in mice with CaMKK β or CaMK IV deletion after stroke.

Conclusions—Our data have established that the CaMKK/CaMK IV pathway is a key endogenous protective mechanism in ischemia. Our results suggest that this pathway serves as an important regulator of blood–brain barrier integrity and transcriptional activation of neuroprotective molecules in stroke. (Stroke. 2013;44:2559-2566.)

Key Words: CaMKK β and CaMK IV • cerebral ischemia

Strokes is the leading cause of disability worldwide. Calcium signaling plays a critical role in the pathology of cerebral ischemia. Limits in the available blood supply lead to energy depletion and uncontrolled release of glutamate, exacerbated by impaired reuptake, and ultimately leads to increased calcium influx through the hyperactivation of the N-methyl-D-aspartate receptor. Increased neuronal-free calcium activates numerous molecules that can participate in signal transduction pathways leading to cell survival or cell death. Many of these secondary effects of calcium are mediated through the ubiquitous calcium sensing protein, calmodulin (CaM). Calcium overload was traditionally thought to be detrimental in stroke. However, clinical trials testing treatments that block calcium failed to show neuroprotection in ischemic stroke. One possible explanation is that those approaches nonspecifically targeted calcium signaling. It has become increasingly recognized that enhancing calcium signaling may also play an important protective role after injury by triggering endogenous neuroprotective pathways.

A critical upstream kinase directly activated by calcium/calmodulin signaling is calcium/calmodulin-dependent protein kinase kinase (CaMKK), a serine/threonine-specific protein kinase. This kinase has 2 isoforms α and β, both of which are expressed in the nervous system and hematopoietic cells. CaMKK expression was also found in endothelial cells. An early study implicated CaMKK in neuronal cell survival pathways. In this study, CaMKK-mediated phosphorylation of Akt protected neurons from apoptosis induced by serum withdrawal in neuroblastoma cells. Once activated, CaMKK phosphorylates its 2 main downstream targets, CaMK I and CaMK IV. CaMK IV is expressed primarily in cells of nervous system, the hematopoietic system, and the gonads. CaMK IV resides in both the nucleus and cytosol but the active form of CaMK IV (phosphorylated) is predominantly nuclear, because of facilitated transportation by importin α. Once CaMK IV is phosphorylated, it enhances the prosurvival B-cell lymphoma protein 2 (BCL2) gene via actions on cAMP response element-binding protein (CREB). CaMK IV also actively regulates...
gene transcription by phosphorylating histone deacetylase 4 (HDAC4). When neurons are under stress, HDAC4 translocates from cytosol into nucleus, then represses the expression of survival gene such as myocyte specific enhancer factor 2. Phosphorylation of HDAC4 by CaMK IV promotes HDAC4 nuclear exports in neurons, resulting in neuroprotection in excitotoxic glutamate condition,7–9 a major cell death mechanism in cerebral ischemia. Interestingly, overexpression of CaMK IV decreased neuronal injury after oxygen glucose deprivation. In contrast, CaMK IV knockdown neuronal injury in mouse cortical neuronal cultures, suggesting that activation of CaMK IV is beneficial to neuronal survival.10 Therefore, CaMKK and its downstream pathways may be endogenous neuroprotective mechanisms in stroke. In the present study, we investigated the role of CaMKK signaling in stroke and its downstream mediators in the ischemic brain.

**Methods**

**Animals**

The present study was conducted in accordance with National Institutes of Health guidelines for the care and use of animals in research and under protocols approved by the Center for Laboratory Animal Care of University of Connecticut Health Center. Both CaMKK β knockout (KO) and CaMK IV knockout mouse were provided by Dr Anthony Means at Duke University and were back-crossed to C57BL/6J background.11 Both KO mice are normal in size and do not display any gross physical or behavioral abnormalities. Control mice for both null lines were generated from F1 heterozygous matings and backcrossed to C57BL/6J backgrounds.12 All studies used male animals age- and weight-matched (21–25 g, 10–12 weeks of age).

**Middle Cerebral Artery Occlusion**

Focal transient cerebral ischemia (90 minutes middle cerebral artery occlusion) was induced in wild-type (WT), CaMKK β KO, or CaMK IV KO mice followed by reperfusion as described previously.13 At the end of ischemia, the animal was briefly reanesthetized, and reperfusion was initiated by filament withdrawal. During the ischemic period, animal body temperatures were controlled at 37°C using a heating pad with feedback thermo-control system (FST). In separate cohorts of CaMK IV KO, CaMKK β KO, and WT animals, as well as the ST0-609 (an inhibitor of CaMKKβ)/vehicle-treated animals (n=4 p/g), physiological measurements including femoral arterial blood pressure, pH, P02, PCO2, and blood glucose were obtained. In those separate cohorts, cortical perfusion using laser Doppler flowmetry was evaluated throughout middle cerebral artery occlusion and early reperfusion as described previously.13 Animals were randomized into stroke and surgical sham cohorts. Investigators who performed the procedures were blinded to drug treatment and genotype.

**Drug Treatment**

ST0-609, a pan CaMKK inhibitor (2 μL, 1.5 mg/mL, dissolved in DMSO) was injected intracerebroventricularly in male WT mice at the coordinates (from bregma; −0.9 mm lateral, −0.1 mm posterior, −3.1 mm deep) 30 minutes before or 2 hours after the onset of middle cerebral artery occlusion. Control animals were injected with equal amount of vehicle (DMSO).

**Results**

**Genetic Deletion of CaMKK β Aggravated Stroke Infarction, Hemorrhagic Transformation, and Edema**

Infarct volumes (percentage of contralateral structure and corrected for edema formation) were significantly higher in CaMKK β knockout mice compared with WT controls 24 hours after stroke (cortex: KO 58.0±7.1% versus WT 39.5±4.0%, P<0.05, striatum: KO 69.6±3.9% versus WT 49.0±6.8%, P<0.05, total: KO 56±5.7% versus WT 40±3.8%, P<0.05, n=7 WT / 8 KO p/g; Figure 1A and 1C). Interestingly, we observed increased rates of hemorrhagic transformation in CaMKK β KO mice after stroke. Macroscopic hemorrhagic transformation was visually identified in brains after 2,3,5-triphenyltetrazolium chloride staining (Figure 1C). Higher hemorrhagic transformation (HT) rates were seen in CaMKK β KO mice (KO 50% versus WT 0%, n=7–8 p/g, P<0.05; Figure 1B). Hemoglobin content was higher in the KO than in WT controls (KO 360±38.6 versus WT 170.0±24.3 μg/sample, n=4 p/g P<0.05; Figure 1D). Mortality rate and neurological deficits scores did not differ between the KO and WT mice after stroke (data not shown). No difference was found in mean arterial pressure, P02, PCO2, or pH between KO and WT controls. In addition, local cerebral blood flow as measured by laser Doppler flow was equivalently reduced during ischemia and restored equally in early reperfusion (ST1).

We measured edema formation at 24 hours after stroke using wet/dry weights, and then calculated the edema index. CaMKK β mice had significantly higher edema formation than WT controls (1.0722±0.0068 versus 1.0426±0.0084, P<0.05 n=6 p/g; Figure 1E).

**Pharmacological CaMKK Inhibition With ST0-609 Exacerbated Stroke Infarct Size**

To confirm the effects of the detrimental effects of CaMKK genetic inhibition, we treated mice with the pharmacological CaMKK inhibitor ST0-609. Treatment of WT mice with ST0-609 (intracerebroventricularly) exacerbated stroke outcome when compared with vehicle treatment 24 hours after stroke (cortex: drug 61.9±5.9% versus vehicle 40.8±4.2% P<0.05, striatum: drug 75.9±4.2% versus vehicle 51.2±4.2% P<0.05, total: drug 59.9±4.2% versus vehicle 42.9±1.9% P<0.05, n=8–9 p/g; Figure 2A). HT rates, mortality rate, and neurological deficits scores did not differ between the drug and vehicle-treated mice after stroke (data not shown). There were no differences in mean arterial pressure, P02, PCO2, or pH between the ST0-609–treated and vehicle-treated groups. In addition, local cerebral blood flow as measured by laser Doppler flow was equivalently reduced during ischemia (drug 13.3±1.0% versus vehicle 12.8±1.2%, n=4 p/g) and was restored equally in early reperfusion (drug 86.9±4.8% versus vehicle 83.1±5.1%, n=4 p/g; ST1).

To verify the mechanism and specificity of ST0-609, we administered ST0-609 (intracerebroventricularly) to CaMKK β KO mice before stroke. ST0-609 had no effect on infarct size in CaMKK β KO mice (cortical ST0-609 54.2±5.1 versus control 59.4±5.6, striatum ST0-609 74.5±5.5 versus control 71.6±4.8, total 53.4±5.0 versus control 56.9±2.7, n=5 p/g; Figure 2B), suggesting that ST0-609 conferred its deleterious effects in stroke at least in part through CaMKK β inhibition.
To determine whether CaMK IV plays protective roles in stroke, we investigated effect of genetic deletion of CaMK IV genetic deletion significantly increased infarct volumes in cortical (KO 64.9±4.9% versus WT 48.9±4.1%, P<0.05), striatal (KO 77.8±5.8% versus WT 62.2±5.7%, P<0.05), and total (KO 61.0±5.1% versus WT 43.4±3.7%, P<0.05) (n=7/pg) compared with WT controls 24 hours after stroke (Figure 3B–3D). This detrimental effect was reflected in the exacerbated neurological deficits of the KO mice (KO 3.1±0.3 versus WT 2.3±0.2, P<0.05). Stroke-induced mortality was seen in 6 of 13 in CaMK IV KO mice, whereas no mortality was seen in the WT controls (P<0.05). Macrophagic cerebral hemorrhagic transformation was visually identified in brains after 2,3,5-triphenyltetrazolium chloride staining or with postmortem inspection in mice that died prematurely after stroke. There is a trend toward statistical significance in HT rates in CaMK IV KO mice when compared with WT controls (KO 53.8% versus WT 14.2%, n=13 in KO, n=7 in WT, P=0.09; Figure 3E).

There were no differences in mean arterial pressure, Po2, Pco2, or pH between CaMK IV KO and WT groups. In addition, local cerebral blood flow as measured by laser Doppler flow was equivalently reduced during ischemia (KO 11.2±1.1% versus WT 12.3±1.1%, n=4/pg) and was restored equally in early reperfusion (KO 77.5±1.0% versus vehicle 83.4±2.0%, n=4/pg; ST1).
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Figure 2. STO-609 increased the stroke infarct volumes in wild-type (WT) mice. A, STO-609 treatment exacerbated stroke injury in WT mice assessed 24 hours after stroke. Calcium/calmodulin-dependent protein kinase kinase (CaMKK) inhibitor was injected intracerebroventricularly in male WT mice 30 mins before the onset of middle cerebral artery occlusion (MCAO). Control animals received the equal amount of vehicle. Cortical, striatal, and total hemisphere infarction volumes were calculated (percentage of nonischemic hemisphere). n=8 vehicle group, n=9 STO-609–treated group. B, CaMKK pan-inhibitor STO-609 did not change the stroke outcome in CaMKK β knockout (KO) mice after 90-minute MCAO. n=5 p/g. STO-609/vehicle were injected in CaMKK β KO mice 30 mins before the onset of MCAO and outcome assessed at 24 hours poststroke. C, Poststroke treatment of STO-609 increased infarcts assessed at 72 hours after stroke. n=8 p/g. STO-609 was injected intracerebroventricularly in male WT mice 30 mins before or 2 hours after the onset of MCAO and stroke outcome was measured at 72 hours’ survival. *P<0.05 vs control (Student t test); data were presented as mean±SEM.

Figure 3. Calcium/calmodulin-dependent protein kinase (CaMK) IV genetic deletion is detrimental in stroke. A, CaMK IV gene deletion increased infarct volume after stroke. Arrow: hemorrhagic transformation (HT); B through D, CaMK IV gene deletion increased infarct volume after stroke. Arrow: hemorrhagic transformation (HT); E, HT rates of CaMK IV knockout (KO) and wild type (WT) controls. CaMK IV KO and WT control mice were subject to 90 minutes of middle cerebral artery occlusion. Cortical, striatal, and total hemisphere infarction volumes (24 hours after stroke) were calculated (percentage of nonischemic hemisphere). *P<0.05 vs control (Student t test); data were presented as mean±SEM. n=7 per group for the infarct data; n=13 in KO, n=7 in WT for the HT rates’ data. Sh indicates sham; and St, stroke.
suggesting that the genetic deletion of CaMKK$\beta$/CaMK IV did not affect brain vascular integrity at baseline level.$^{18}$

**CaMKK**$\beta$**KO and CaMK IV KO Mice Had Reduced Phosphorylated Histone Deacetylase 4 Levels**

HDAC4 nuclear translocation has been shown to be detrimental for neuronal survival.$^{10}$ CaMK IV inhibits HDAC4 translocation in neurons under stress via phosphorylation of HDAC4.$^{10}$ We observed that stroke significantly reduced phosphorylated histone deacetylase 4 (pHDAC4) levels in WT controls (Figure 5, $P<0.05$). This reduction was further exacerbated in both CaMKK$\beta$ KO ($P<0.05$ KO stroke versus WT stroke, $n=2$ in sham, $n=4$ in stroke) and CaMK IV KO mice ($P<0.05$ KO stroke versus WT stroke, $n=2$ in sham, $n=3$ in stroke) 6 hours after stroke when compared with WT controls (Figure 5).

**CaMK IV KO Mice Had Reduced BCL2 Levels 6 Hours After Stroke**

Expression of BCL2 is regulated by HDAC4 and is neuroprotective in stroke models. We examined BCL2 levels in CaMK IV KO mice. In stroke mice, BCL2 levels were significantly lower in CaMK IV KO mice when compared with WT controls (Figure 6, $n=2$ in sham, $n=4$ in stroke, $P<0.05$ KO stroke versus WT stroke). Surprisingly, BCL2 levels did not differ between the CaMKK$\beta$ KO and WT controls at 6 hours after stroke (data not shown).

**CAMKK**$\beta$**Inhibition With STO-609 Had No Effects on Phosphorylated AMPK Levels**

CaMKK is a known upstream kinase for AMPK,$^{19}$ a kinase implicated in pathology of stroke.$^{19}$ We examined the effect of CaMKK inhibition on phosphorylated AMPK (pAMPK) levels 4 hours after stroke. Stroke led to an increase in the levels of pAMPK; however, STO-609 treatment did not change pAMPK levels (Figure I in the online-only Data Supplement, $n=3$ p/g), suggesting CaMKK signaling in stroke may be independent of AMPK.

**Discussion**

This study made the following significant novel findings. First, we found inhibition of CaMKK either pharmacologically or genetically was detrimental in cerebral ischemia and identified CaMKK as a novel biological target for stroke treatment. Second, we showed that CaMKK$\beta$ deletion reduced phosphorylation of nuclear CaMK IV, a major downstream molecule of CaMKK. In addition, we found that CaMK IV deletion also exacerbated infarct size. Third, loss of either CaMKK$\beta$ or CaMK IV perturbed BBB integrity after stroke, as reflected by a significant increase in edema formation and MMP activation. Fourth, CaMKK/CaMK IV pathway inhibition resulted in transcriptional inactivation, as indicated by changes in HDAC4 and BCL2 levels in KO mice after stroke. Finally, our work demonstrated that inhibition of CaMKK did not affect AMPK phosphorylation, indicating CaMKK operates independently of AMPK signaling in cerebral ischemia.

The exacerbated stroke outcome in CaMKK$\beta$ KO mice and in STO-609-treated WT mice suggested that CaMKK normally mediates neuroprotective signaling. CaMKK resides in both the cytosol and the nucleus where it responds to changes in Ca$^{2+}$ levels and signals CaM-kinase. CaMKK is held in an inactive state by its autoinhibitory domain, which interacts
with the catalytic domain to prevent kinase activity. Binding of Ca\textsuperscript{2+}/CaM releases this autoinhibitory domain, thus activating the kinase. CaMKK then activates its 2 primary downstream targets CaMK I and CaMK IV through phosphorylation.\textsuperscript{9} The neuroprotective actions of CaMKK signaling seen in our study may be mediated in part through CaMK IV as loss of CaMK IV–aggravated stroke injury and led to a significant increase in mortality rate. Deletion of CaMK IV led to an exacerbation in stroke injury to a greater degree than CaMKK inhibition, as CaMK IV KO mice had increased mortality rate in addition to significantly larger infarcts. It is likely CaMKK inhibition with selective deletion of the \( \beta \) isoform or STO-609 treatment did not completely block the downstream CaMK IV activity, therefore its effect on stroke outcome was less than the complete loss of CaMK IV. CaMK IV promotes neuronal survival and inhibits apoptosis in cell models possibly through enhancing CREB phosphorylation.\textsuperscript{19} Loss of CaMKK \( \beta \) or CaMK IV results in decreased CREB phosphorylation, a neuronal survival factor, in cerebellar granule cell neurons, and re-expression of CaMKK \( \beta \) or CaMK IV in granule cells that lack CaMKK \( \beta \) or CaMK IV, respectively, restores CREB phosphorylation WT levels.\textsuperscript{12} Overexpression of CaMK IV reduced mouse cortical neuronal injury after oxygen glucose deprivation in vitro whereas knockdown CaMK IV aggravated cell injury and the effect of CaMK IV was attributed to subsequent regulation on CREB phosphorylation.\textsuperscript{11} The results were consistent with those of our in vivo study. Chen et al\textsuperscript{20} conducted the first in vivo study to examine the functional role of CaMK IV in brain ischemia and demonstrated that KN-93 (a CaMK inhibitor) enhanced global ischemic injury in rats. However, the data have to be interpreted with caution as KN-93 is not a specific CaMK IV inhibitor, but instead a more selective inhibitor of CaMK II,\textsuperscript{21} which seems to have no direct interaction with CaMKK or CaMK IV.

A novel, but likely critical function of CaMK IV signaling has recently been identified. CaMK IV regulates the function of HDAC4, a newly identified contributor to cell death.\textsuperscript{10} HDAC4 is usually trapped in the cytosol, possibly because of its binding to the 14-3-3 protein family.\textsuperscript{22} However, active shuttling of HDAC4 between the cytosol and nucleus can be induced. Once in the nucleus, HDAC4 deacetylates histone, making it inaccessible to the transcriptional machinery thus reducing gene transcription.\textsuperscript{23} Phosphorylation of HDAC4 recruits 14-3-3 protein resulting in nuclear exportation,\textsuperscript{10} thereby inhibiting its own effects on transcriptional repression. It is hypothesized that HDAC4 represses the transcription of key endogenous survival factors including myocyte specific enhancer factor 2 and CREB, which increases transcription of a variety of prosurvival genes, including Bcl-2.\textsuperscript{24} HDAC4 accumulates in nucleus after toxic glutamate exposure. Interestingly, CaMK IV phosphorylates HDAC4, efficiently exporting this protein from nucleus.\textsuperscript{10} To the best of our knowledge, we were the first to report a stroke-reduced HDAC4 phosphorylation, indicating a compromise in gene transcription of survival factor under ischemic condition.

Figure 5. Calcium/calmodulin-dependent protein kinase kinase (CaMKK) \( \beta \) knockout (KO) and CaMK IV KO mice further exacerbated the reduction of phosphorylated histone deacetylase 4 (pHDAC4) levels 6 hours after stroke. A and C, CaMKK \( \beta \) deletion reduced pHDAC4 levels in mice. B and D, CaMK IV deletion reduced pHDAC4 levels in mice. Brains were collected 6 hours after onset of middle cerebral artery occlusion or sham operation. pHDAC4 levels were assessed with Western blot. Each band represents 1 mouse brain. *\( P \textless 0.05 \): sham vs stroke; \#\( P \textless 0.05 \): KO vs WT (1-way ANOVA). HDAC4 indicates histone deacetylase 4.
The HDAC4 phosphorylation reduction was further exacerbated in both CaMKK β KO and CaMK IV KO mice. BCL2, a downstream molecule of HDAC4/CREB was also reduced in CaMK IV KO mice after stroke, although not in the CaMKK β KO mice at this specific time point. Perhaps it was because only 1 CaMKK isoform was deleted or because CaMKK is the upstream kinase of CaMK IV and its effect on BCL2 expression may be more delayed than CaMK IV deletion. Therefore, regulating HDAC4 activity may be a mechanism by which CaMK IV contributes to endogenous neuroprotection in stroke.

The CaMKK complex has also been implicated in BBB integrity, a disruption of which is 1 of the important contributing factors to edema development, brain injury, and hemorrhagic transformation. Interestingly, the activation CaMKK inhibits the maturation and retards the differentiation of neutrophils in mouse myeloid cell lines. As neutrophils are a major source of MMP-9 in the ischemic brain, CaMKK may be an important endogenous protector of the BBB by reducing neutrophil egress and MMPs into the brain parenchyma. Interestingly, we did not observe increased HT in STO-609–treated mice after stroke. There may be a difference between acute and chronic inhibition of CaMKK, as an acute dose of STO-609 may not have immediate effects on neutrophils maturation and differentiation. Additionally, STO-609 was delivered directly to the brain through intracerebroventricularly. A central versus peripheral difference may also exist in the effects of CaMKK inhibition in stroke outcome. Furthermore, HDAC inhibition, a capacity that CaMKK/CaMK IV seems to possess, is also thought to be able to inhibit MMPs in ischemia brain via an nuclear factor–xβ–dependent mechanism. However, this is less likely, given the fact that pharmacological inhibition with STO-609 did not induce significantly higher HT in stroke. Nevertheless, systemic inhibition of CaMKK/CaMK IV may exacerbate MMP activity in stroke. Indeed, in our studies, we found that mice with deletion of the CaMKK β isoform or CaMK IV had greater MMP activity as early as 6 hours after stroke. Of note, the observed MMP activity change is more likely to be mechanistic than merely a correlation with infarct size, as at this early time point stroke is not yet mature. Additionally, endothelial cells, an important component of the neurovascular unit, also express CaMKK and may play a role in the enhanced HT observed in our work as well; however, this mechanism remains speculative at this point as the role of CaMKK in endothelium under ischemic stress is still unclear. Accordingly, we saw a greater degree of edema formation in CaMKK β KO mice, which is a consequence of BBB breakdown and important contributor to patient mortality. We also observed high hemorrhagic transformation rates in mice lacking CaMKK β (statistically significant) or CaMK IV (a trend toward significance). This suggests that CaMKK/CaMK IV signaling may protect the BBB from injury after stroke. Future studies to investigate the role of endothelial CaMK cascade in stroke, MMPs, the regulation of CaMKs on neutrophil activation, and egress in stroke are warranted.

We initially thought that loss of CaMKK may reduce stroke injury because of its potential inhibition of AMPK activation, as it is a major upstream activator (via phosphorylation) of AMPK. We have previously found that activation of AMPK is deleterious in stroke, and inhibition, either with genetic or pharmacological methods, reduced injury. However, it seems that loss of CaMKK signaling leads to significant damage, and this is independent of AMPK signaling as stroke-induced activation of AMPK was unchanged in mice treated with STO-609. During ischemia, other AMPK upstream kinases such as LKB1 may function as regulators for AMPK activity.

In conclusion, we demonstrated the detrimental effects of inhibition of CaMKK after stroke. Our data showed that CaMK IV may be an important mediator of CaMKK’s effect in cerebral ischemia. Our work suggests CaMKK or CaMK IV are potential therapeutic targets for stroke treatment.

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Disclosures
None.

References
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SUPPLEMENTAL MATERIAL

Inhibition of CaMKK β and CaMK IV is detrimental in cerebral ischemia

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Supplemental Methods:

Infarct measurement

At 24 h or 72 h after stroke, histological assessment was performed. Briefly, the animals were sacrificed; the brains were immediately removed, and cut into 5 individual 2-mm slices. Brain slices were stained with 1.5% 2,3,5-triphenyltetrazolium (TTC) at 37 °C for 8 mins and were fixed with 4% formalin. Images were digitalized, and the infarct volumes (corrected for edema) were analyzed using computer software (Sigmascan Pro5) as previously described.

Western blots

Six hours after the onset of cerebral ischemia, mice were euthanized, brains were homogenized using RIPA lysis buffer (Cell signaling). BCA assay (Pierce) was conducted to measure protein concentration of the samples. Then protein was loaded on a 4% to 15% gradient sodium dodecyl sulfate–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. pHDAC4, BCL2, pAMPK was probed with antibody from Cell Signaling (1:1000), pCaMK IV and Collagen IV was probed with antibody from abcam (1:500 abcam). β-actin (1:5000; Sigma) or histone (1:5000, sigma) was used as a loading control. Blots were incubated overnight in primary antibody at 4°C in Tris-buffered saline containing 4% bovine serum albumin and 0.1% Tween 20. Secondary antibodies (goat antirabbit IgG 1:5000 for pAMPK, goat antimouse IgG 1:5000 for β-actin; Chemicon) were diluted and an electrochemiluminescence (Pico) detection kit (ThermoScientific) was used for signal detection.

Subcellular fractionation

Their brains were rapidly removed and flash frozen in 2-methyl butane on dry ice then stored at -80 °C. Samples were homogenized using dounce homogenizers with cold lysis solution (10 mmol/L Tris–HCl, pH 7.5; 5 mmol/L MgCl2; 0.1 mmol/L EDTA; 1.5 mmol/L CaCl2; 0.25 mmol/L sucrose; 1 mol/L DDT; 10 % Triton X-100; 1:50 protease inhibitor). Homogenates were centrifuged at 800 g for 10 min at 4 °C. The pellet which contained the nuclear fraction was suspended in lysis buffer and run through a sucrose gradient composed of 1.8 and 2.3 mol/L sucrose with ultracentrifugation at 30,000 g for 45 min. The extracted pellet was transferred into nuclei pure storage buffer (Sigma-Aldrich) and centrifuged at 2,300 rpm for 10 min. The nuclear pellet was resolved with extraction buffer (Sigma-Aldrich), sonicated for 10 s three times, and stored at -80 °C. The nuclear samples were then used for Western blots. Each sample point reflects pooled samples (2 brains/sample).

Behavior measurement
Neurological deficits were scored as described previously. 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; and 4, no spontaneous locomotor activity or barrel rolling.

**Hemoglobin assay**

At 48h after MCAo, WT and CaMKK KO mice were perfused transcardially until the outflow fluid from the right atrium was colorless. Brains were rapidly harvested and dissected into right and left hemispheres. The hemispheres with stroke were then homogenized and sonicated in distilled water, followed by 30-minute centrifugation (13 000 rpm). Then 50 µl supernatant was mixed with 200 µl reaction reagent (QuantiChrom Hemoglobin Assay Kit; BioAssay Systems, Hayward, Calif, USA). After 15 min, optical density was measured by a spectrophotometer (Wallac 1420, PerkinElmer, Waltham, MA, USA) at 405 nm. Total hemoglobin content was calculated as micrograms per samples.

**Edema formation**

At 24 h of stroke, the brain was quickly removed after sacrifice for edema measurements. The brain was blotted to remove residual absorbent moisture, and separated into right and left hemispheres. The wet weight was determined with a resolution of 0.1 mg. The dry weight was measured after the hemispheres are dried for 3 days at 100 °C in a drying oven. The tissue water content was then calculated as % H₂O = (1 − dry wt/wet wt) × 100%.

**Gelatinase activity assay**

The activity of MMP-9 and MMP-2 were determined by gelatin zymography. After brains were removed and homogenized using RIPA lysis buffer (Cell signaling) aliquots of the supernatant, containing 500 ug proteins were subjected to affinity precipitation with gelatin-conjugated sepharose beads (GE, Life Science). The bound material was released from the beads in 50 µl elution buffer of 10% DMSO. Then samples were analyzed with 10% gelatin zymogram gel (Bio-rad).

**Statistics analysis**

Data from individual experiments were presented as mean±SEM. One way ANOVA with post-hoc (Bonferroni correction for multiple comparisons whenever appropriate) was used for the comparison of the means between the experimental groups except the neurological deficit scores, which were done by Mann-Whitney U test, and proportion comparison, which was done by two-tailed Z-test. P<0.05 was considered statistically significant. Behavioral and histological assessments were done by an investigator blinded to genotype/drug treatment.

**Supplemental Tables**
Supplemental table I STO-609 treatment and CaMK IV gene deletion did not alter physiological parameters and local cerebral blood flow

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Supplemental table I There were no significant differences in physiological parameters either between the STO-609 treated mice and their corresponding vehicle controls or between the two strains of KO mice (CaMK IV KO/CaMKK β KO) and their corresponding WT controls. The physiological parameters were measured before and 60 minutes after the onset of MCAO. Cortical cerebral blood flow was measured using LDF throughout the ischemic period and 30 mins into reperfusion. n=4 p/g. Mean±Sem. Units for pCO2, pO2 and MABP is mmHg; for glucose, it is mg/dl.
**Supplemental Figure I** CaMKK β KO and CaMK IV KO mice show no difference in baseline Collagen IV levels when compared to WT controls. Collagen IV levels were assessed with Western blot in mice no stroke brains tissue. A and B: Representative Western blots of Collagen IV; C and D: Quantified densitometry data. C: n=3p/g; D: n=5 WT/ n=3 KO.
Supplemental Figure II CaMKK β inhibition with STO-609 did not reduce pAMPK levels after stroke. CaMKK inhibitor (2 μl, 1.5mg/ml, dissolved in DMSO) was injected introcerebroventricularly (icv) to male WT mice 30 mins prior to the onset of MCAO. Control animals received the equal amount of vehicle. Brains were collected 4 hours after onset of MCAO or sham-operation. pAMPK levels were assessed with Western blot (n=3 p/g).